RESEARCH COMMUNICATION

Evaluation of Primers and PCR Performance on HPV DNA Screening in Normal and Low Grade Abnormal Cervical Cells

Arkom Chaiwongkot[1], Chamsai Pientong[1], Tipaya Ekalaksananan[1], Bunkerd Kongyingyoes[2], Jadsada Thinkhamrop[3], Pissamai Yuenyao[3], Supannee Sriamporn[4]*

Abstract

High risk human papillomaviruses (HR-HPVs) are associated with increased risk of normal cervical cells developing to dysplasia and cervical carcinoma. Therefore, HR-HPV DNA testing can predict an endpoint of cervical carcinogenesis that is earlier than the development of cervical abnormalities. Not only the sensitivity of methods but also the amount of HPV DNA are very important and might be parameters to distinguish HPV detection. In this study, we evaluated the effects of primer sets and the polymerase chain reaction (PCR) performance with low viral load samples with normal cervical cytology (140 samples) and mild dysplasia (140 samples) using two consensus primers MY09/MY11 and GP5+/6+. The PCR was performed with single and nested PCR. Positive samples with both primer sets were then HPV genotyped by dot blot hybridization. Results showed higher sensitivity of single PCR using primer GP5+/GP6+ than primer MY09/MY11. HPV DNA was detected in 15% (21 of 140)and 20.7% (29 of 140) of normal cervical samples, respectively. For mild dysplasia samples, HPV DNA was detected in 37.1% (52 of 140) with MY09/MY11 and 50% (70 of 140) using GP5+/GP6+. In normal cervical samples, the positivity rate was increased to 38.5% (54 of 140) by nested PCR using primer GP5+/6+, but only 2 mild dysplasia samples that were negative by single GP5+/6+ were positive by auto-nested PCR. These results suggested that, in low viral load samples, the sensitivity of HPV DNA detection depends not only on primer sets but also PCR performance. HPV 16 was the most common in mild dysplasia samples (20.8%), whereas HPV type 58 was found in 11.1%. This study suggested that nested PCR might be necessary for HPV DNA detection in cervical samples of women participating in cervical cancer screening.

Key Words: Abnormal cervical cells - HPV DNA screening - primer sets - PCR performance

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Introduction

HR-HPVs infections of the genital organs have been established as causative agent of cervical cancer and its precursor lesion, cervical intraepithelial neoplasia (CIN). Screening of cervical cancer cells using cytology based method or Papanicolaou (Pap) staining has been considered the most successful cancer screening program. However, the estimated true sensitivity of the conventional Pap test is on the order of 50% to 60% in the routine screening setting (Fahey et al., 1995; Nanda et al., 2000). Introduction of liquid based cytology (LBC) has increased the sensitivity and reduced the number of false positive results of cancer cases when compare to conventional Pap smear. However, even such improved cytology tests may miss 15% to 35% of CIN III or cancer in a routine screening setting (Solomon et al., 2001; Kulasingam et al., 2002). There are some limitations of cytology based method with its low sensitivity and presumptive diagnosis. Moreover, it is not effective in diagnosis of HPV infection, that is detected in >99% of cancers. Therefore, the successful cervical cancer screening strategies have been developed including HPV testing.

There are vast majority of HPV infections in women population around the world range from 2% to 44%. This variation can be explained by differences in the age range of the populations studied and the sensitivity of the HPV DNA detection assay (Walboomers et al., 1999). In cervical level, numerous qualitative techniques based on molecular hybridization or nucleic acid amplification technique such as PCR have been used to detect HPV DNA (Hubbard et al, 2003). An analytical sensitivity of techniques varies widely from low (e.g. 0.1 copy/cell for Southern blot) to high sensitivity (e.g.10-5 to 10-6 copy/cell for PCR). As for PCR, its sensitivity depends on primers (e.g. MY11/09 versus GP5/6), DNA polymerase or size of the amplified target DNA (Snijders et al., 2003).

PCR has been widely used and shown to be the most sensitive method for detection of HPV infection in clinical samples. Number of primers including, consensus primers and type-specific primers have been used for the detection of HPVs. However, consensus primers (MYO9/

*[1]Dept.of Microbiology, [2]Dept.of Pharmacology, [3]Dept. of Obstetrics and Gynecology, Faculty of Medicine, Khon Kaen University,, [4]College of Medicine and Public Health, Ubon Rajathanee University, Thailand *For Correspondence: supannee0911@yahoo.com*

MY11 and GP5+/6+) that amplified the most conserved L1 region have been widely used in clinical and epidemiological studies. The positive rate of HPV DNA detection is influenced not only by primer sets and DNA polymerase but also by whether PCR is single or nested (Remmerbach et al., 2004).

Previous studies revealed that the effect of HR-HPV infection on CIN development is strongly influenced by viral genome copy numbers (Ylitalo et al., 2000; Sun et al., 2001). Low viral loads are found with normal cytology with a median HR-HPV load of 69 (1.4-3442) pg/mL by Hybrid Capture II, whereas in cases (ASCUS and LGSIL) the mean was 132 (1.8–2393) pg/mL. Longitudinal analysis performed on follow-up samples also showed that progression to CIN2/3 is linked to increase in HPV16 burden, whereas in controls a decrease of at least 1 log HPV16 DNA load was observed over ≥2 time points. This information demonstrated the kinetics of HPV load (Monnier-Benoit et al., 2006) and reflected the fact that the amount of HR HPV DNA increases with the grade of the lesion (Swan et al., 1999; van Duin et al., 2002; Weissenborn et al., 2003; Lillo et al., 2005). However, there may be no significant association of viral load with invasive cancer (Wu et al., 2006). Detection of HR HPV DNA using PCR is considered to be potentially useful as a primary screening test, solely or combination with a Pap smear to detect cervical cancer precursors. Overall cytology at the level of ASCUS or worse had a sensitivity of 55% (95%CI: 51-59%) compared to 96% (95%CI: 94-97%) for HPV testing. Moreover, the sensitivity of cytology is highly variable, ranging from 19-76%, whereas that for HPV testing is uniformly high (range 85-100%) (Cuzick et al., 2006). Fluctuation in viral load below the detection threshold of screening tests can lead to misclassification of some infected women as HPV negative.

The purpose of the present study was to explore the effect of primer sets and PCR type on HPV DNA detection in cervical samples containing different viral loads. Cervical samples diagnosed by Pap test as normal cytology and mild dysplasia including ASCUS and LSIL samples were selected, alng with MYO9/MY11 and GP5+/6+ primer sets and single and nested PCR for comparison.

Materials and Methods

Study subjects

Normal cytology samples (140) were obtained from women participating for cervical cancer screening at department of Obstetrics and Gynecology, Srinagarind Hospital, Khon Kaen University (Thailand) and determined by cytologist at the Department of Pathology, Faculty of Medicine, Khon Kaen University. Cervical samples with mild dysplasia (140) were collected from women who were referred to colposcopic examination by Gynaecologists. All samples were collected between November 2003 and December 2005.Written informed consent was obtained from the patients by the participating gynaecologists. The study was approved by the local ethics committees of the Khon Kaen University, Khon Kaen, Thailand.

Sample preparation

Cervical cells were washed in 5 ml of 1X phosphate buffered saline twice by centriguation at 2000 rpm for 10 minutes and DNA was extracted from pellets using the PUREGENETM DNA extraction kit (Gentra) according to the manufacturer's instructions. Integrity of the DNA was confirmed by amplification of a housekeeping gene (beta-globin) from the DNA samples.

HPV DNA detection using single step and nested PCR amplification

All samples were tested for HPV DNA by single PCR with primers pairs of MYP9/MY11 and GP5+/6+ respectively. The amplification mixture of 50 ul PCR for MY09/MY11 primer set contained 1XPCR buffer, 2 mmole $MgCl_2$, 0.2 mmole of each dNTP, 50 pmole of each primer and 1.25 U Taq DNA polymerase. Amplifications were performed for 40 cycles with the following parameters; initial denaturing at 95°C for 5 minutes; each cycle at 95°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute; final extension at 72°C for 10 minutes. The amplification mixture of 50 µl PCR for GP5+/6+ primer set contained 1XPCR buffer, 3 mmole $MgCl_2$, 0.2 mmole of each dNTP, 50 pmole of each primer and 1.25 U Tag DNA polymerase. Amplifications were performed for 40 cycles with the following parameters; initial denaturing at 94°C for 4 minutes; each cycle at 94°C for 1 minute, 42°C for 1 minute and 72°C for 30 seconds; final extension at 72°C for 4 minutes. 5 ul of PCR product was electrophoresed through 1.5 % agarose gel and visualized under an ultraviolet transilluminator.

For the nested PCR, the HPV DNA negative samples were re-amplified using the GP5+/6+ primer using 50 ul mixture contained 1XPCR buffer ,3 mmole $MgCl_2$, 0.2 mmole of each dNTP, 50 pmole of each primer and 1.25 U Tag DNA polymerase. Amplifications were performed for 30 cycles with the following parameters; initial denaturing at 94°C for 4 minutes; each cycle at 94 °C for 1 minute, 42°C for 1 minute and 72°C for 30 seconds; final extension at 72 °C for 4 minutes.

HPV genotyping

The samples identified as positive for HPV DNA were genotyped by dot blot hybridization as follows: Dot blot hybridization for nested GP5+/6+ positive samples. All samples with HPV DNA positive by PCR using nested GP5+/6+ primers were HPV genotyped by dot blot hybridization. The probe for each HPV type was prepared by biotinylated GP5+/6+ PCR product of 11 HPV plasmids (11, 16, 18, 31, 33, 35, 39, 45, 51, 52 and 58). PCR products of each positive sample was denatured and applied to nylon membranes (Biodyne B) by dot blotting (Bio-Rad, Hercules, Calif.) and hybridized at 42°C overnight with biotinylated GP5+/6+ PCR product probe (500 ng/ml). Following washing at 60°C to remove nonspecifically bound probe, bound probe was detected with streptavidin-horseradish peroxidase (Zymed) and chemiluminescence detection kit (LumiGLO, KPL). Membranes were use to expose Kodak Medical X-ray film and HPV positivity was determined by establishment of a negative cutoff and signals above the cutoff were positive.

Dot blot hybridization for MY09/11 positive samples

12 biotin labeled oligonucletide probes were used for detection of HPV-6/11(MY12), -16(MY95), -18(MY130), -31(MY92), -33(MY16), -35(MY115), -39(MY89), -45(MY69), -51(MY87), -52(MY81), -58(MY94) and 68(MY19Atrogen Life Technologies) (Bauer et al, 1992) in positive samples by MY09/11 primers. PCR products of each positive sample was denatured and applied to nylon membranes (Biodyne B) with a dot blot apparatus (Bio-Rad, Hercules, Calif.) and hybridized with 3 pmoles/ml of probe at 42°C overnight. High stringency washing was then performed as described above. HPV positivity by dot blot was determined as above.

Sequencing

PCR positive samples that were negative by dot blot hybridization were genotyped by direct sequencing. PCR products were purified with a BILATEST® PCR cleanup kit (Bilatec, Viernheim, Germany) and submitted to the Molecular Informatic Laboratory (Hong Kong) for sequencing.

Statistical analysis

The correlation of the results of the HPV DNA detection obtained with both primers pairs was analyzed by McNemar's chi-square test ($p<0.05$). The kappa statistic was calculated to evaluate the agreement between rates of HPV detection by both primers pair. In general, kappa values of 0, 0.01-0.2, 0.21-0.4, 0.41-0.6, 0.61-0.8, 0.81-0.99 and 1.0 indicate poor, slight, fair, moderate, substantial, almost perfect and perfect agreement, respectively.

Results

HPV DNA detection

HPV DNA was detected in 15% and 20.7% of normal cervical samples, respectively, using MY09/MY11 and GP5+/GP6+ PCR, and in in 37.1% and 50% of mild dysplasia samples. There was a fair and substantial agreement between both primer sets (kappa values of 0.236 and 0.629) as shown in Table 1. A significant difference was found in mild dysplasia samples with single PCR using MY09/11 and GP5+/6+ primers pair ($p<0.05$) (Table 1).

Percentage HPV DNA detection was increased to 38.5% (54 of 140) with normal cervical samples by auto-nested PCR using GP5+/6+. There was a statistical significance ($P<0.05$) with moderate agreement (kappa value of 0.588) between single and nested PCR results (seeTable 2). There were only 2 mild dysplasia samples that were negative by single GP5+/6+ which became positive with nested PCR . No significant difference was showed with almost perfect agreement (kappa value of 0.97) between single and nested PCR results (Table 2).

HPV genotyping

HPV DNA positive samples of normal cytology (54 samples) and mild dysplasia (72 samples) were genotyped. HPV 16 was the most common in both (seeTable 3).

Table 1. HPV DNA Amplification using Single MY09/11 and GP5+/6+ Primers in Normal Mild Dysplasia Cervical Samples

		GP5+/6+ results		
MY09/11 results		HPV positive	HPV negative	Total
Normal	HPV positive	9	12	21
	HPV negative	20	109	119
	Total	29	111	140
Agreement 78.7%		Kappa statistics	0.236	
Dysplasia	HPV positive	48	4	52
	HPV negative	22	66	88
	Total	70	70	140
Agreement 81.4 %		Kappa statistics	0.629	

Table 2. HPV DNA Amplification using Single and Auto-nested PCR GP5+/6+ Primers in Normal and Mild Dysplasia Cervical Samples

		Single GP5+/6+ results		
Nested GP5+/6+		HPV positive	HPV negative	Total
Control	HPV positive	29	25	54
	HPV negative	0	86	86
	Total	29	111	140
Agreement 82.1%		Kappa statistics	0.588	
Dysplasia	HPV positive	70	2	72
	HPV negative	0	68	68
	Total	70	70	140
Agreement 98.6 %		Kappa statistics	0.97	

Discussion

In our study, the MY09/MY11 PCR was less sensitive than the GP5+/6+ PCR. This may explain that primers MY09/11 are the degenerate primers that can amplify multiple HPV infection and used high annealing temperature (55°C). They therefore has less efficiency in amplify some HPV types such as HPV 35. For consensus primers GP5+/6+, annealing temperature was used at 42°C. This advantage is that they can amplify the single HPV infection better than multiple infections (Qu et al., 1997). Qu et al (Qu et al., 1997) compared between MY09/11 and GP5+/6+ primer systems in clinical samples containing with multiple HPV infections. The results showed that HPV DNA were detected in 90% and 47% using MY-PCR and GP+-PCR respectively. This reflected the sets of primers MY09/11 that can amplify multiple type of HPV DNA. In our study, most HPV positive samples showed single infections. This result may reflect

Table 3. Proportions of HPV Genotypes

HPV Type	Mild dysplasia	Normal cytology
16	15 (20.8 %)	15 (27.7%)
58	8 (11.1%)	2 (3.7%)
11	7 (9.7%)	3 (5.5%)
18	5 (6.9%)	14 (25.9%)
33	2 (2.7%)	3 (5.5%)
6/30/35/39/43/45/51/52/56/59/66/70		
Each	1 (1.3%)	-
16/18	3 (4.1%)	3 (5.5%)
16/33	2 (2.7%)	-
58/33	1 (1.3%)	-
Unidentified	14 (19.4%)	14 (25.9%)
Total	72	54

from PCR product of GP5+/6+ primer set. However, there were unclassified HPV types (table 3) that might be mixed infection. Remmerbach et al (2004) investigated HPV DNA in oral and cervical samples using single step PCR of MY09/11 and GP5+/6+ primer pairs and found MY09/11 PCR to be much less sensitive. In cervical samples, HPV DNA was detected in 33.9% and 46.4% by single PCR using MY09/11 and GP5+/6+ respectively.

When auto nested GP5+/6+ PCR was applied for detection of HPV DNA in cervical cells in order to increase the sensitivity of the detection, there was an increase in the detection in women with normal cytology from 20.7% to 38.5%. In mild dysplasia samples, only 2 samples were positive that the detection rate increase from 50% to 51.4%. This result suggested not only the type of primer but amount of HPV DNA in samples were necessary for sensitivity of HPV detection. In this study, auto-nested PCR increased the sensitivity of HPV DNA detection in sample that suspected to contain low viral load. Remmerbach et al(Remmerbach et al., 2004) performed auto nested PCR of GP5+/6+ primer system that increased the positivity of HPV DNA detection from 35.8% to 65.1% in oral samples. For cervical samples, HPV DNA was increased from 46.4% to 69.6%. This results support that nested PCR can increase the sensitivity of HPV DNA detection in samples with low concentration that found in women population with normal cytology than precancerous lesions. van Duin M et al (van Duin et al., 2002) showed that women with CIN II/III had a significantly higher viral load than women with CIN ≤ 1. Sun et al(Sun et al., 2001) showed increase in viral load correlated with lesion grade and lesion size from women with no visible, small and large lesions. One explanation to support the advantage of nested PCR is that the detection of amplified products by gel electrophoresis and ethidium bromide staining may be limited if PCR product is too small. Gallo et al (Gallo et al., 2003) showed that southern blot hybridization can detect PCR product that undetectable by EtBr staining because of the small amounts of DNA. Nested PCR decreased the risk of false negatives because it can amplify small amounts of PCR product of the first round of PCR. Although HPV DNA testing is more sensitive than cytology, false negative can occur in case of low concentration of viral DNA in clinical samples.

In conclusion, this study suggested that the more sensitive test such as nested PCR should be applied for HPV DNA detection as adjunct test in cervical cancer screening program.

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